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ORIGINAL PAPER

# Separation of long DNA fragments by inversion field capillary electrophoresis

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**Abstract** This study reports improved pulsed field capillary electrophoresis (PFCE) for separation of large DNA ladders. Important analytical conditions, including gel polymer concentration, ratio of forward to backward pulse duration, and separation potential, were investigated for their effects on the separation performance of DNA ranging in size from 0.1 to 10.0 kilo base pairs (kbp). Results show that DNA fragments from 0.1 to 8.0 kbp can be resolved with high resolution, simultaneously, in a short time. The ratio of forward to backward pulse duration affects the separation performance for DNA fragments greater than 1.5 kbp, and 3 or 4 is the optimum value of the ratio for separation of DNA up to 10 kbp. Furthermore, the separations that were obtained with 74–19,329 bp λ-DNA restriction fragments clearly demonstrate a dramatic improvement in the separation time and resolution over the conventionally used square-wave PFCE. The inversion field capillary electrophoresis reported here may help enable future DNA analysis studies to be performed quickly and effectively.

**Keywords** Capillary electrophoresis · Electrophoresis · DNA separation · HEC · Pulsed field capillary electrophoresis · Inversion field capillary electrophoresis

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**Abbreviations**

| | |
|---|---|
| CE | Capillary electrophoresis |
| HEC | Hydroxyethylcellulose |
| kbp | Kilo base pairs |
| PFCE | Pulsed field capillary electrophoresis |
| PFGE | Pulsed field gel electrophoresis |
| TBE | Tris-borate–EDTA |

## Introduction

Analysis of DNA fragments by CE with a constant electric field is a versatile means of DNA diagnosis in biochemistry and molecular biology [1–5]. However, many experiments indicate that in high electric fields, large DNA fragments are trapped, which leads to broad electrophoretic bands and loss of separation [6, 7]. This is because the process of migration of long DNA fragments in a sieving matrix is closely related to both the electric field applied to the capillary and the Kuhn length of the polymer molecule. Moreover, the biased reptation with fluctuations model has

pointed out that for DNA shorter than a certain length $N_k$*, the mobility will be proportional to the chain length; for nucleic acids longer than $N_k$*, mobility will depend only on the electric field and the pore size of the polymer network [8, 9].

In order to resolve long DNA fragments efficiently, Carle et al. introduced a pulsed electric field into gel electrophoresis (PFGE) [10, 11]. Experiments have demonstrated that high-frequency pulses eliminate the molecular trapping, thus the separation of large DNA fragments was achieved [12]. With advances in separation technology, pulse electric field was applied to capillary electrophoresis, which is an attractive alternative to PFGE for single-strand and double-strand DNA analysis because it has numerous advantages including better separation performance and better efficiency in terms of time and labor [13–15]. In recent reports, a pulse electric field was also combined with microchip electrophoresis [16]. In addition, electrophoretic imaging analysis [17, 18] has shown that the molecule moves in the sieving matrix by changing the coil, U (or V)-shaped or stretched conformations. And most importantly, in pulsed-field conditions, the lifetime of the U conformation is proportional to DNA size and inversely proportional to the fragment free velocity. Therefore, the separation of long fragments is improved dramatically.

Several conditions affect separation performance in PFCE. The forward, $V_f$, and backward, $V_b$ separation potentials, and the times of the forward, $t_f$, and backward $t_b$, separation potentials are global electrophoretic conditions for DNA separations, especially the backward electric field, $V_b$, which promotes DNA entanglement and causes secondary entanglement of long DNA fragments during migration. The average applied electronic field corresponds to the applied field of constant-field capillary electrophoresis. Another important electric condition is pulse frequency. It determines the separation performance by affecting the orientation and reorientation process of DNA fragment. For example, in the separation of DNA fragments between 0.5 and 2.0 kbp, an electric field frequency more than 30 Hz cannot improve the DNA separation, and electrophoretic peaks in PFCE suffer from strong tailing [19]. In addition, different separation potential waveforms, for example chirping mode with increasing frequency or sine wave electric field, etc., were expected to improve long DNA separations. However, the limiting case is when using sine wave electric field capillary electrophoresis for separation, sometimes the number of peaks obtained is more than that of the DNA fragments [20, 21]. Therefore, it is necessary to find a more effective waveform of PFCE for DNA separation with a wider range.

Whereas PFCE stimulated separation of long DNA fragments, the physical properties of the sieving polymer, including the mesh size and the formation of physical network, are also important in determining separation performance [22]. The network size in the sieving polymer solution, which is related to the sieving polymer concentration, is the dominant factor for the DNA entanglement in PFCE. Therefore, both the electric field condition in PFCE and the sieving polymer concentration dominate the orientation and/or reorientation process of the DNA fragments, especially the long DNA fragments.

Our previous research [23] investigated square-wave PFCE. It showed that in this type of PFCE, the separation of long DNA (>1.5 kbp) fragments was successfully carried out with high modulation depth, yet it was impossible to optimally separate DNA of all sizes with high resolution simultaneously, which hindered its application. In an attempt to resolve DNA fragments (0.1–10.0 kbp) simultaneously with high resolution, we investigated the separation of DNA fragments by improved square-wave PFCE, which was based on $V_f = V_b$ but $t_f \neq t_b$. In order to distinguish it from the traditional square-wave PFCE, we refer to it as inversion field capillary electrophoresis (IFCE).

## Materials and methods

### Chemicals

All chemicals reagents were used without further purification. Hydroxyethylcellulose (HEC, 1,300 K) was purchased from Sigma (St Louis, MO, USA). SYBR Green I was purchased from Invitrogen (Carlsbad, CA, USA). Tris-borate-EDTA (TBE, 10×) buffer was from Bio-Rad (Hercules, CA, USA). HEC polymer solution containing 1× SYBR green I was prepared by dissolution in the 0.5× TBE buffer. DNA ladders (0.1 kbp and 1.0 kbp), λ-DNA, and EcoT14 I restriction enzyme were bought from Takara (Shiga, Japan). All were kept frozen at −20 °C before use. 10× H buffer consisted of 500 mmol $L^{-1}$ TBE, 100 mmol $L^{-1}$ $MgCl_2$, 10 mmol $L^{-1}$ dithiothreitol, and 1,000 mmol $L^{-1}$ NaCl, which were purchased from Takara.

### Apparatus

The IFCE system designed and built in our laboratory has previously been described in detail [23]. Briefly, the 75 μm ID, 365 μm OD fused-silica capillary tube (Polymicro Tehchnologies, Phoenix, AZ, USA) had an effective length of 8 cm and a total length of 15 cm. To minimize electroosmosis, the inner surfaces were coated with polyacrylamide by use of Hijerten's method [24, 25]. The coating process has been described in detail in Ref. [26]. Capillary electrophoresis was performed with a system consisting of a microscope with epi-illumination (IX71; Olympus, Tokyo, Japan). To produce a pulsed-field electric field, a high-voltage power supply (model 610E; Trek,

Medina, NY, USA) was used. Waveform and the conditions for determination of the pulsed field were controlled by LabVIEW software (National Instrument). The excitation wavelength from a mercury lamp was filtered by use of an optical filter (U-MWIB-3; Olympus) to furnish 460–495 nm, which was the wavelength of the excitation maximum of the conjugate of SYBR Green I and the nucleic acid. The fluorescence signal was detected by use of a photomultiplier (R928; Hamamatsu Photonics, Hamamatsu, Japan). The signal was digitized by use of a National Instrument PCI-6024E (Austin, TX, USA). DNA samples diluted with 0.5× TBE were electrokinetically injected at 100 $V cm^{-1}$ for 2 s. The entire detection system was enclosed in a dark box.

λ -DNA restriction enzyme reaction process

The reaction mixture was prepared by mixing 19 μL distilled water, 2 μL 10× H buffer, 1 μL λ-DNA sample, and 2 μL EcoT14 I restriction enzyme to give a final volume of 25 μL. The solution was mixed uniformly by vortex mixing (Scientific Industries, Germany), and centrifuged to spin down all DNA reaction sample by use of a model 1910 centrifuge (Kubota, Tokyo, Japan) for 1.0 min, and the mixture was then incubated in a 37 °C water bath for 1 h to stimulate the restriction enzyme reaction with the DNA. When the reaction was complete, 2 μL 0.1 mol $L^{-1}$ EDTA solution was added into the sample to stop the reaction; samples were then stored at −20 °C.

## Results and discussion

CE versus IFCE for separation of large DNA fragments

IFCE was performed under conditions in which the absolute magnitude of forward potential and backward potential was the same (for example, $V=V_f=V_b=1{,}500$ V), but the duration of the forward time ($t_f$) and backward time ($t_b$) was different (for example, $t_f=40$ ms, $t_b=10$ ms). In such case, the average separation potential was calculated as 900 V on the basis of the equation:

$$V_{average} = V(t_f - t_b)/(t_f + t_b) \quad (1)$$

We first compared the migration behavior of 0.1–10.0 kbp DNA fragments in 0.3% HEC (1,300 K) by CE and IFCE. As shown in Fig. 1, the constant field CE is mainly effective for DNA fragments smaller than 1.0 kbp. However, when the separation decreased from 1,500 V (Fig. 1a) to 900 V (Fig. 1b), the resolution for DNA fragments greater than 1.0 kbp improved not so much although the analysis time was extended. For comparison, Fig. 1c shows that under inversion field conditions, the separation performance for the DNA sample was substan-

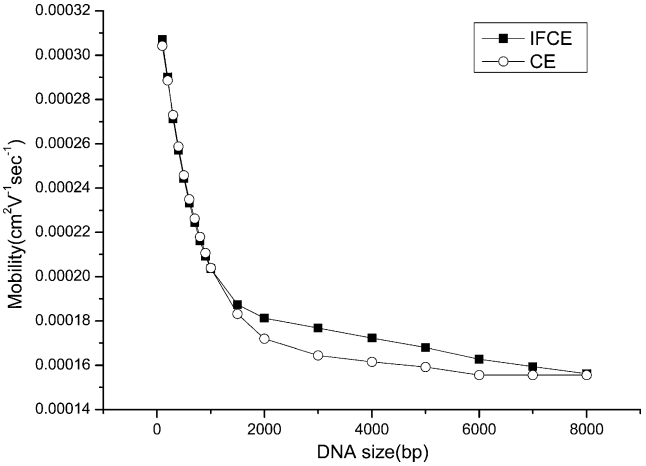


**Fig. 2** Mobility of DNA versus its length by IFCE ($V_f=V_b=1{,}500$ V, $t_f=40$ ms, $t_b=10$ ms), and CE (900 V)

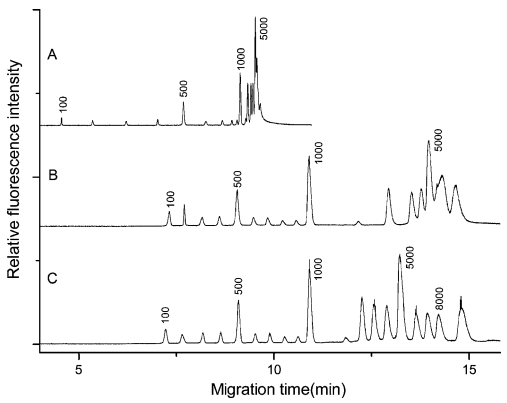


**Fig. 1** Electropherogram of the DNA (0.1–10.0 kbp) fragments in 0.3% HEC (1300 K) by (**A**) CE: 1,500 V; (**B**) CE: 900 V; (**C**) IFCE: $V_f=V_b=1{,}500$ V, $t_f=40$ ms, $t_b=10$ ms at room temperature, loadings: 100 $V cm^{-1}$ (2 s)

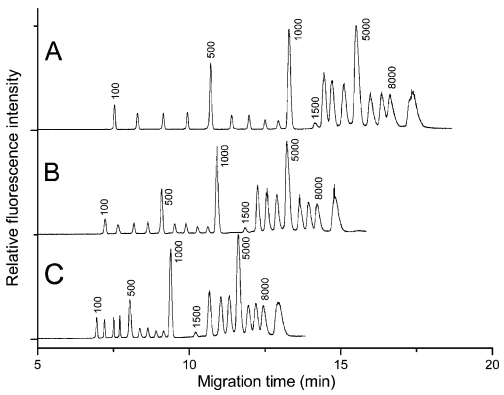


**Fig. 3** Electropherogram of DNA with different concentrations of HEC (1,300 K) polymer: (**A**) 0.4%, (**B**) 0.3%, (**C**) 0.2%; Separation conditions: $V_f=V_b=1{,}500$ V, $t_f=40$ ms, $t_b=10$ ms, other conditions are the same as in Fig. 1

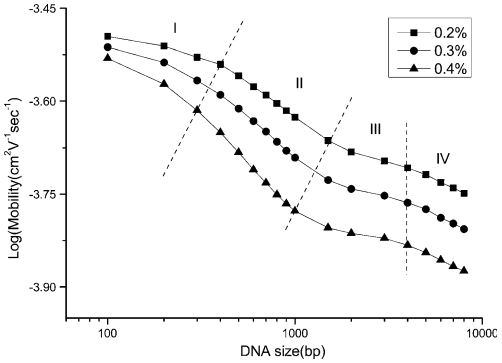


**Fig. 4** Dependence of DNA mobility on chain length in different concentrations of HEC polymer by IFCE

tially improved. Moreover, compared with the conventional square-wave PFCE [23], the resolution for DNA fragments between 0.5 and 1.0 kbp was improved.

Figure 2 illustrates the mobility of DNA fragments under IFCE conditions and constant-field conditions. The mobility under IFCE conditions was defined as the ratio of average velocity to average electric field strength, which is analogous to the mobility of DNA in constant-field CE [27].

$$\mu = \frac{v_{average}}{E_{average}} = \frac{L_e/T}{V_{average}/L_t} = \frac{L_e L_t}{TV\left(t_f - t_b\right)/\left(t_f + t_b\right)} \qquad (2)$$

where $v_{\text{average}}$ and $E_{\text{average}}$ are the average values of migration velocity and electric field strength, respectively. $T$ is the migration time of each fragment, $L_e$ is the effective capillary length, and $L_t$ is the total length of the capillary. Data in Fig. 2 shows that the mobility of DNA fragments smaller than 1.0 kbp in IFCE was identical with the mobility in direct field CE. However, DNA fragments greater than 1.0 kbp migrated faster under pulsed-field conditions than under constant-field conditions. This is probably because the DNA fragments (<1.0 kbp) were so short that they were not easily affected by the inversion electric field. Moreover, the separation of DNA fragments from 1.0 to 8.0 kbp was improved dramatically because of the decrease in mobility spread.

## Effect of concentration of polymer

DNA separation with different concentration of sieving polymer was performed under the pulsed-field conditions of $V_f$=$V_b$=1,500 V, $t_f$=40 ms, $t_b$=10 ms. Figure 3 shows that with increasing polymer concentration, the DNA fragments migrate slowly because of the smaller mesh size of the polymer network, and DNA fragments over 2.0 kbp were well recognized in each polymer concentration. Moreover, four regimes were observed in the double logarithmic plot of DNA size against mobility. Figure 4 shows that DNA fragments move with the same trend for each concentration of polymer. The migration processes of DNA fragments under 3.0 kbp were followed for reasons of explanation under DC conditions [28]. For 0.4 kbp to 1.5 kbp DNA fragments in 0.2% HEC and 0.3 kbp to 1.0 kbp in 0.4% HEC, DNA fragments migrate linearly with increasing DNA molecule size. Because the polymer mesh size was dependent on sieving polymer concentration, the slope of the approximate line in the secondary regime tilted strongly in 0.4% polymer concentration. For 1.5 kbp to 3.0 kbp DNA fragments in 0.2% HEC and 1.0 kbp to 3.0 kbp DNA fragments in 0.4% HEC, the slope became smaller. For DNA fragments above 3.0 kbp, the slope was increased, and this was a unique phenomenon observed in this pulsed field experiment. Interestingly the magnitude of the slope for every polymer concentration was almost equal. This behavior was never observed in DNA separation under DC conditions.

## Effect of modulation depth and separation potential

Modulation depth is defined as the ratio of forward to backward pulse durations in IFCE. For example, if the

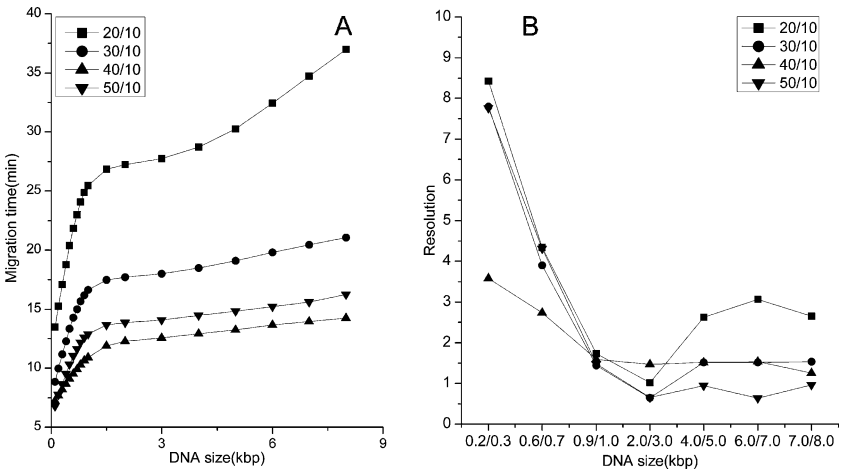


**Fig. 5** The separation performance for DNA (0.1–10.0) fragments by IFCE with different ratios of forward to backward pulse durations: (**A**) the migration time; (**B**) the resolution. The right edge number represents $t_f/t_b$; for example, the first number indicates the forward time is 20 ms and the backward time is 10 ms. Separation conditions: $V_f$=$V_b$= 1,500 V, other conditions are the same as in Fig. 1

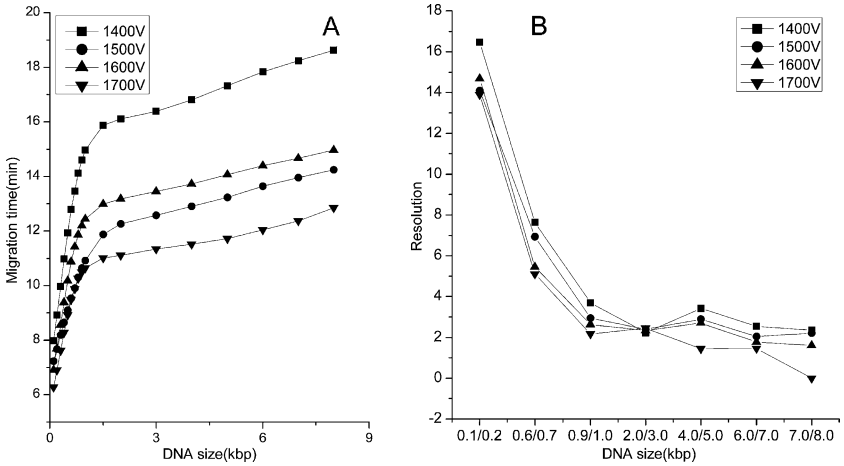


**Fig. 6** The separation performance for DNA (0.1–10.0) fragments by IFCE with different forward to backward pulse electric fields: (**A**) the migration time; (**B**) the resolution; Separation conditions: $t_f$=40 ms, $t_b$= 10 ms, other conditions are the same as in Fig. 1

modulation ratio is 1, the DNA cannot migrate, because the average separation potential is zero. Thus if one wants to force the DNA molecule to move forwards, the ratio of forward to backward pulse durations should be higher than 1.0. In order to investigate the effect of forward and backward duration on the DNA separation performance by IFCE, we separated the DNA fragments in 0.3% HEC (1,300 K) polymer with $V_f$=$V_b$=1,500 V by use of different field pulses in which the ratio of forward to backward duration was 2, 3, 4, or 5. Figure 5 shows the analysis time for DNA (0.1–10.0 kbp) (Fig. 5a) and the dependence of resolution for adjacent DNA fragments on the ratio of forward to backward pulses (Fig. 5b) by IFCE. Data in Fig. 5 reveal that increasing the ratio of forward to backward pulses leads to short run time and a decrease in resolution of adjacent DNA fragments. This is because the effective separation potential is linearly increased with the ratio of forward to backward pulse duration. However, an increase in effective electric field strength possibly leads to an increase in peak dispersion because of increased Joule heating [29]. As shown in Fig. 5a, the analysis time was reduced from 40 min to 15 min when the ratio of pulse durations varied from 20:10 to 40:10, yet the resolution is still greater than 1.0. Therefore, the ratio of times of 3:1 or 4:1 for the forward and backward directions gives the best separation performance for DNA (0.1–10.0 kbp) fragments.

We also investigated the effect of separation potential on resolution. DNA fragments were separated in 0.3% HEC (1,300 K) with 40-ms forward pulses and 10-ms backward pulses with different separation potential. Figure 6 shows that for DNA fragments smaller than 2.0 kbp, separation potential has very little effect on the separation. However, for DNA molecules longer than 2.0 kbp, resolution deteriorates with increasing separation potential. We also found that for higher separation potential, the peak width was broad if the forward and backward separation potential was higher than 1,500 V. This is probably because with growth of the separation potential, the driving force for the DNA molecule will be increased. Consequently, DNA fragments moved more rapidly and the mobility difference between the adjacent DNA fragments was reduced, thus the DNA fragments migrated together. Meanwhile, Joule heating is in proportion to the separation potential [30], so increasing the separation potential will increase Joule heating in the polymer. Furthermore, Joule heating may reduce the viscosity [31] of polymer and therefore it may strengthen DNA diffusion during the run. As a result, resolution of the DNA fragments declined. So in subsequent work we used 1,500 V as forward and backward separation potential and duration ratio 3 as optimized IFCE condition.

## Separation of λ-DNA restriction fragments by IFCE

We found that the optimized conditions for DNA fragments between 0.1 and 10.0 kbp was 0.3% for HEC polymer concentration, 3 for the ratio of forward to backward

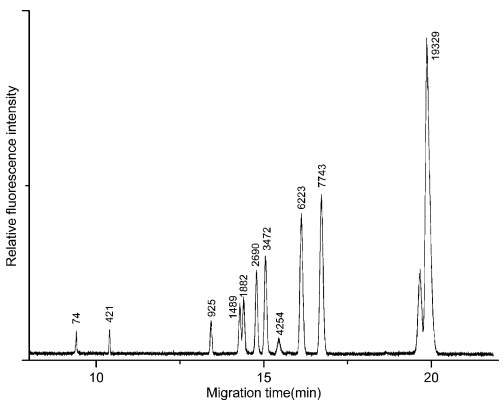


**Fig. 7** Separation of λ-DNA fragments digested by EcoT14 I restriction enzyme with IFCE at room temperature in 0.3% HEC (1,300 K), 0.5× TBE buffer. Separation conditions: $V_f$=$V_b$=1500 V, $t_f$=40 ms, $t_b$=10 ms, loading: 100 V cm$^{-1}$ (20 s)

duration, and 1,500 V for the separation potential. Here we separated the λ-DNA fragments restricted by EcoT14 I restriction enzyme, and the sizes of the gene fragments were 74, 421, 925, 1,489, 1,882, 2,690, 3,472, 4,254, 6,223, 7,743, and 19,329 bp. A total of 11 λ-DNA fragments ranging from 74 to 19,329 bp were clearly observed by IFCE (Fig. 7), except that the last fragment was split into a sub-peak [32].

## Conclusions

In the work discussed in this paper we demonstrated the separation of long DNA fragments (0.1–10.0 kbp) by IFCE with modulated forward and backward pulse durations. The results showed that the analysis time was reduced whereas the separation efficiency was maintained. We also investigated the effects of polymer concentration, ratio of forward to backward duration, and effective separation potential on separation performance. Result shows that 0.3% HEC (1,300 K), $V_f = V_b = 1{,}500$ V and ratio of forward to backward pulse duration of 3 or 4 result in the best separation performance for DNA fragments over the whole range. Furthermore, the restriction λ-DNA fragment was baseline resolved under the optimized conditions. And we believe that this approach can provide the solution for the separation of long DNA fragments.

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